ANTINEOPLASTIC EXTRACT FROM *ACHILLEA MILLEFOLIUM*

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to isolated and purified plant extracts, and more particularly to one from *Achillea millefolium* to treat and prevent neoplastic disorders.

(b) Description of Prior Art

Yarrow is an important member of the *Asteraceae* branch of the *Compositae*, the daisy family. Common names for yarrow include milfoil staunch weed, nosebleed, soldier's herb, carpenter's wort, thousand weed, woundwort, bloodwort boomadaran and knight's milfoil. There are about 100 different species of yarrow that grow mainly in temperate region of the world. Yarrow, or *Achillea millefolium*, is said to have been used by the Greek hero Achilles to stop the bleeding of his warrior's wounds.

Yarrow (*Achillea millefolium* LINNAEUS) is used as a medicinal plant in different parts of the world, as an haemostatic, emmenagogue, antipyretic and diaphoretic in cases of common cold.

An infusion is generally made from *Achillea millefolium*, which is also used for lack of appetite, cramps, flatulence and other stomach-related disorders. Aboriginal people and pioneers also used yarrow as a tea to treat digestive disorders and fevers and as a poultice to treat cuts and burns, and chewed the leaves to relieve toothache pain. Yarrow has long been associated with the healing of wounds and the steeming of blood flow. The existing literature indicates that yarrow improves colon and liver function, is good against anemia, liver disease, skin disease, eczema, liver, psoriasis and rashes, as well as for treating cold, flu, fever, hypertension, painful menstruation

and bleeding. The value of yarrow as an anti-spasmodic and diuretic agent, as well as an anti-inflammatory and antiseptic compound, has been demonstrated.

The use of yarrow tea against cancer is known. For example, in Iran, people have been using yarrow tea for cancer for several hundreds years. Yarrow tea has been used in different parts of the world for centuries without manifesting toxicity or side effects, and some cancer patients in the United States and Canada have been taking yarrow as an alternative medicine. However, no proven anticancer activity has been reported.

Antitumor sesquiterpenoids were recently identified and isolated as methyl esters from *Achillea millefolium*, namely achimillic acids A, B, and C,. These compounds are active against mouse P-388 leukemia cells *in vivo*.

Known constituents of yarrow are essential oils, namely cineol, proazulene and achilleine.

Neoplastic disorders such as cancer are treated with agents which are generally toxic with severe side-effects.

It would be highly desirable to be provided with a substantially pure biologically active fraction isolated from *Achillea millefolium* that would have an antineoplastic activity, and that could be used to treat or prevent diseases such as cancer.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide purified biologically active fractions isolated from *Achillea millefolium* that may be used to treat or prevent disorders such as cancer.

In accordance with the present invention there is provided a substantially pure biologically active

extract isolated from *Achillea millefolium*, said extract having an antineoplastic activity.

In accordance with one embodiment of the present invention, the extract consists of a crude methanol extract.

In accordance with another embodiment of the present invention, there is provided the use of such an extract for the preparation of a medicament for the treatment and/or prevention of a neoplastic disorder, such as cancer.

In accordance with another embodiment of the present invention, there is provided an antineoplastic composition to treat and/or prevent cancer, said composition comprising a therapeutically effective amount of a substantially pure extract isolated from *Achillea millefolium* having antineoplastic activity, and a suitable carrier.

In accordance with another embodiment of the present invention, there is provided a method for treating and/or preventing a cancer in a patient, said method comprising administering to said patient a therapeutically effective amount of a substantially pure biologically active extract isolated from *Achillea millefolium* with a pharmaceutically acceptable carrier.

The composition may be administered to a patient susceptible of developing or suspected of having a cancer, in an amount efficient to treat or prevent the cancer.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates the tracing obtained with the analytical HPLCs of the extracts;

Fig. 2 illustrates the fractions obtained with a large scale;

Fig. 3 illustrates a dose-response relationship for a methanol extract; and

Fig. 4 illustrates a dose-response relationship for fractions of methanol extracts.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there are provided purified biologically active fractions isolated from *Achillea millefolium* to treat diseases such as cancer.

Fractions from *Achillea millefolium* LINNAEUS have been isolated. The purified fractions were administered to animals in which cancer was induced. No toxicity was observed at the doses administered. Moreover, the isolated organic soluble fractions have antimetastatic activity in a mouse cancer model. The isolated active fractions contain biologically active molecules that may be used to treat diseases including cancer.

More particularly, the crude methanol fraction had a good antimetastatic activity in the Lewis lung carcinoma model.

The animal model published by Tozyo et al. (*Chem Pharm Bull*, 1994, **42**:1096-1100) consists of a mouse leukemia P388 cell model. Tozyo et al. (supra) injected both cells and drugs intraperitoneally. This does not mimic physiological/pharmacological conditions observed in human cancer. Indeed, the conditions in Tozyo et al. resemble that of a petri dish where both the target and the drug are in direct contact. According to the present invention, the cells are injected subcutaneously to the Lewis lung carcinoma model. The cells then invade a distant site, such as lung, and form metastases. The test article is given by intraperitoneal route. Accordingly, the active

component(s) need to be absorbed, perhaps metabolized, before acting on primary tumors and/or metastases. This is closer to human disease in term of the growth versus multistep mechanisms of invasion.

As may be seen in Fig. 3, a dose-response relationship was observed.

As may be seen if Fig. 4, the E1, E2 and E4 fractions were the most active in inhibiting lung metastases.

Molecule(s) responsible for the biological activity of the extracts may be identified and characterized. The(se) molecule(s) may then be used to treat or prevent cancer, leukemias, as well as other diseases.

The fractions and molecules contained therein are advantageous over the whole plant or teas made from the plant.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

EXAMPLE I

Fractionation

Dried plant was grounded, and then stirred in methanol at 25°C for 48h. The resulting extract was filtered and treated with fresh methanol for another 48h. The combined extracts were filtered, evaporated and analyzed by HPLC. Analytical HPLC (Waters™ 600, Photodiodearray™ 996) was performed with two Whatman Partisil™ 10 ODS-2 analytical columns in series (4.6 x 250 mm). The gradient used consisted of 25-100% acetonitrile in water, 50 min gradient at a flow rate of 1 ml/min. Three fractions were identified according to retention times, namely the fractions 0-10, 11-22

and 23-60. The tracing of this analytical HPLC is shown in Fig. 1.

A large scale was then used. Briefly, 2 grams from methanol extract were dissolved in glass-distilled methanol and filtered, and three separations were performed with one Partisil™ 10 ODS-2 MAG-20 preparative column (22 x 500 mm) with the following gradient: 25-100% acetonitrile in water, 50 min. gradient at a flow rate of 18 ml/min. Four fractions were collected for each injection according to the following retention times: F1: 4.63-15.9; F2: 15.9-24.4; F3: 24.4-40.2; and F4: 40.2-60. The fractions are shown in Fig. 2.

The fractions were freshly solubilized in ethanol (final concentration is less than 20% of distilled water), and immediately used for *in vivo* studies or stored at -80°C.

EXAMPLE II

Lewis lung carcinoma (LLC) cell line and cell culture

The Lewis lung carcinoma (LLC) clone, M47, with a high metastatic potential to the lung, was established and characterized (Brodt P, *Cancer Res.*, **46**: 2442, 1986). These cells were confirmed free of mycoplasma infection. Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin, under 5% CO_2. Cells were passaged twice a week. Stocks of cells were generated and stored as early passages (passage no. 8-10 received as passage no. 1, was considered the initial stock). Cells were then propagated and stocks of the same passages were established and stored in liquid nitrogen for further experiments.

For tumor induction, cells were grown to 70% confluence in complete medium and then collected using

trypsin-EDTA solution [0.05% trypsin, 0.53 mM EDTA-4Na in HBSS without Ca^{++}, Mg^{++}, and $NaHCO_3$; Cellgro no. 25-052-Li]. Cells were then centrifuged and washed three times with phosphate buffer solution [D-PBS, Ca^{++} and Mg^{++} free; Cellgro no. 21-031-LV], and resuspended at a dilution of 0.1-1 x 10^6 cells/0.1 ml. Viability was examined by Trypan blue staining and only flasks in which the viability was >95% were used for *in vivo* studies.

The C57BL/10 mouse strain from the research laboratories and incinerators was used, and access to the animal facility is strictly limited to animal users. The animal room has two doors, one serving as the entrance and the other providing direct access to washing, sterilization and incineration facilities, which allows an accurate adjustment of environmental parameters including temperature, humidity, ventilation and lighting.

EXAMPLE III

Tumor cell inoculation and treatment

Five mice were housed per cage and fed a diet of animal chow and water *ad libitum*. After one week of acclimatization, LLC cells were transplanted subcutaneously, as a suspension of tumor cells (2-5 x 10^5 viable cells/0.1 ml) in the axillary region of the right flank. Animals were subjected daily to general examination. Tumor growth was monitored every second or third day using calipers. Tumor were measured along the longest axis (length) and the perpendicular shortest axis (width) and the relative tumor volume (in cm^3) was calculated by the formula: [Length (cm) x (width cm)2]/2. When the tumor reached a size of 0.5-1.0 cm^2 (in approximately 2-3 weeks), the mice were randomized into three groups.

In the first group, the mice were subjected to surgery to remove the primary tumor. The mice were lightly anesthetized with Forane. The skin overlying the tumor was cleaned with betadine and ethanol in a laminar flow hood. A small skin incision (0.5-1.0 cm) was made using a sterile scalpel and the tumor was carefully separated from the normal tissues (skin and muscle). LLC (at an early stage of growth; 1-3 weeks) is a well-localized tumor, and separation was easy to achieve without any significant damage to normal tissues. The tumor was removed, weighed and fixed for histopathology purposes. The wound was closed with surgical stainless steel clips (Autoclips™; 9 mm; Clay Adams, Inc., Parsippany, NJ). The site was further disinfected with Betadine™ and the animal was housed as described earlier.

In the second group, the mice were randomized after surgery into groups of 5 per cage. The cages were randomly assigned to specific experimental groups. The mice were then labeled by numbers using the "ear punching" method. Mice were checked daily to ensure the absence of infection. Animals with disconfort were sacrified immediately. An additional extra-group of control mice was included to determine the optimal timing for sacrifice in order to obtain a significant number of well localized lung metastases. The second group was subjected to the same experimental procedure as the first group, with the exception of drug treatment. Based on the second group, a period of two weeks after removal of the primary tumor was sufficient to obtain an average of 20-30 nodules on the lung surface. Therefore, a two-week period after primary tumor removal was used to sacrify treated mice.

EXAMPLE IV

Dosing schedule and treatment

Drugs were given by intraperitoneal (ip) route (0.5 ml per animal) in daily administration after tumor cell inoculation. Control animals were given the same volume of saline solution (0.9% sodium chloride; Abott Laboratories, lot no. 12 455 WS). The dose of each drug was normalized to an average of 20 g/body weight/per animal. The schedules for drug treatment were based upon conditions described in Figs. 3-4.

EXAMPLE V

Animal sacrifice, tumor/organs preparation

At the end of each experiment, for a total of 5-8 weeks, animals were sacrified in a CO_2 chamber and autopsied. Tumors, organs or both were removed under sterile conditions using a laminar flow hood. Tumors were weighed. Organs (5/group) were examined for gross pathological changes and then fixed in 10% formalin. Lungs were fixed in 10% Bouin's fixative diluted in a formalin solution, and lung surface metastases were counted using a stereomicroscope at 4x magnification or a magnifying-glass.

EXAMPLE VI

Statistical analysis

The umpaired Student t-test was used to compare statistical significance among various groups.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following,

in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.